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Transport stress affects the fecal microbiota in healthy donkeys

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Abstract

Background: With the development of large-scale donkey farming in China, long-distance transportation has become common practice, and the incidence of intestinal diseases after transportation has increased. The intestinal microbiota is important in health and disease, and whether or not transportation disturbs the intestinal microbiota in donkeys has not been investigated.

Objectives: To determine the effects of transportation on the fecal microbiota of healthy donkeys using 16S rRNA sequencing.

Animals: Fecal and blood samples were collected from 12 Dezhou donkeys before and after transportation.

Methods: Prospective controlled study. Cortisol, ACTH, and heat-shock protein 90 (HSP90) concentrations were measured. Sequencing of 16S rRNA was used to assess the microbial composition. Alpha diversity and beta diversity were assessed.

Results: Results showed significant ($P < .05$) increases in cortisol (58.1 ± 14.6 to 71.1 ± 9.60 ng/mL), ACTH (163.8 ± 31.9 to 315.8 ± 27.9 pg/mL), and HSP90 (10.8 ± 1.67 to 14.6 ± 1.75 ng/mL) on the day of arrival. A significantly lower ($P = .04$) level of bacterial richness was found in fecal samples after transportation, compared with that before transportation without distinct changes in diversity. Most notably, donkeys had significant decreases in *Atopostipes*, *Eubacterium*, *Streptococcus*, and *Coriobacteriaceae*.

Conclusions and Clinical Importance: Transportation can induce stress in healthy donkeys and have some effect on the composition of the in fecal microbiota. Additional studies are required to understand the potential effect of these microbiota

Abbreviations: HSP90, heat-shock protein 90; LEfSe, linear discriminant analysis (LDA)-effect size; OTU, operational taxonomic unit; PCoA, principal coordinate analysis.

Guimiao Jiang and Xinhao Zhang contributed equally to this study.

changes, especially significantly decreased bacteria, on the development intestinal diseases in donkeys during recovery from transportation.

KEYWORDS
donkey, fecal microbiota, hormonal changes, transport stress

1 | INTRODUCTION

Domestic animals are transported for various reasons, including breeding, slaughter, and biomedical purposes, and also is associated with animal welfare, food safety, marketing, and trade barriers.[1-3] Transportation exposes animals to various potential stress factors, such as upload and offload, vehicle bumps, crowding, noise, temperature changes, as well as food and water deprivation.[4,5] These factors contribute to weight loss, affect the quality of animal products,[6] increase physical injury and tissue damage, attenuate immune function, increase susceptibility to disease,[7,8] and even may lead to death.[9] Increasing evidence indicates that transport stress has caused substantial economic losses to the animal industry worldwide.[6,10]

The bacterial microbiota is complex and plays a key role in human and animal health. Imbalances in the microbial communities can be associated with a wide variety of diseases in the equine gastrointestinal tract, including colitis,[11] laminitis,[12] equine grass sickness,[13] and transient diarrhea in foals.[14] Previous studies have shown that transportation can alter the composition and total population of gut microorganisms. One study examined effects of transportation on fecal bacterial microbiota in healthy horses and reported a significantly lower abundance of *Clostridiales*.[15] Another study found that 2 hours of travel disturbed the fecal microbial ecosystem in horses, which could increase the risk of triggering microbial dysbiosis in the hindgut.[16] However, reports of the effects of transportation on the gastrointestinal microbiota of donkeys are relatively sparse. In recent years, transporting donkeys from traditional donkey-concentrated areas for fattening and breeding has become a major breeding model in China and has been accompanied by an increase in long-duration transportation. Previous data showed that gastrointestinal problems in the long haul transport of horses are very common, accounting for 27% of transportation issues,[17] and thus gastrointestinal problems induced by transportation also might be a threat to the health of transported donkeys. Because of the relationship between animal health and the gastrointestinal microbiota, it is critical to understand the impact of transportation on the gut microbiota. We evaluated the effects of transport on the fecal bacterial microbiota of donkeys using high-throughput pyrosequencing, which could give a new insight into the pathophysiology of diseases related to gastrointestinal microbiota during recovery from transportation.

2 | MATERIALS AND METHODS

This study was approved by the Ethics Committee for Laboratory Animal Care (Animal Ethics Procedures and Guidelines of China) at the

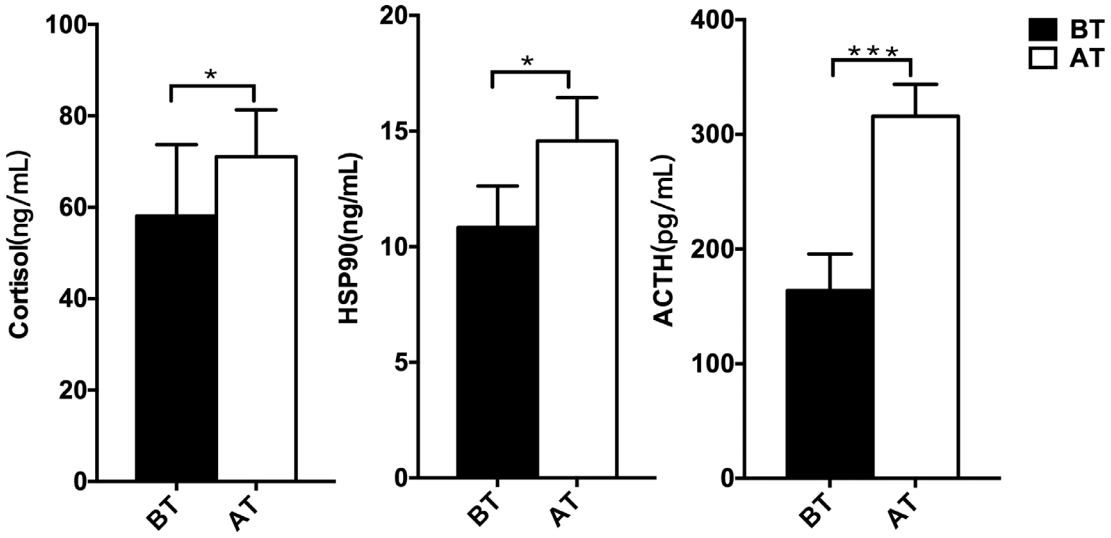

FIGURE 1 Changes in the concentrations of plasma Cor, HSP90, and ACTH before (BT) and after transport (AT). Data are shown as means, and error bars represent the standard deviation (SD) (n = 12). *P < .05, ***P < .001

Shenyang Agricultural University (Permit No. 264SYXK<Liao>2011-0001, September 2018).

2.1 | Animals and transport

Dezhou donkeys are an indigenous breed unique to China. Healthy male Dezhou donkeys were selected from Inner Mongolia Dong-E Black Donkey Animal Husbandry Co., Ltd, in Chifeng City, Inner Mongolia Province, China. The selected donkeys were bought and then transported to a private breeding farm (Dong-E E-Jiao Co., Ltd, Shandong Province, China) that produces Dezhou donkeys.

Twelve male Dezhou donkeys were used for the study. Median body weight (BW) was 140.0 kg (range, 132.0-148.5 kg) and median age was 11 months (range, 10-12 months). The donkeys were clinically healthy and provided free access to water and feed composed of hay and carbohydrate commercial concentrates daily. None of the donkeys had previously experienced road transport. During transportation, the average environmental temperature and humidity were −10°C and 28%, respectively. The surrounding walls of the truck (13.4 m long and 5.6 m wide) were equipped with iron guardrails, and the floor was iron with extremely thin bedding materials. The truck did not have roof coverings, and the donkeys therefore were exposed to different weather conditions. The transport started from Chifeng City in Inner Mongolia Province at 17:00 hours and the animals arrived at Dong-E City in Shandong Province the next day at 14:00 hours, which represents a distance of 950 km and a travel time of approximately 21 hours, including 10 stops (≤20 minutes) for observation of conditions and to provide supplementary feed and water. The routes were secondary roads and expressways. Before (BT) and after

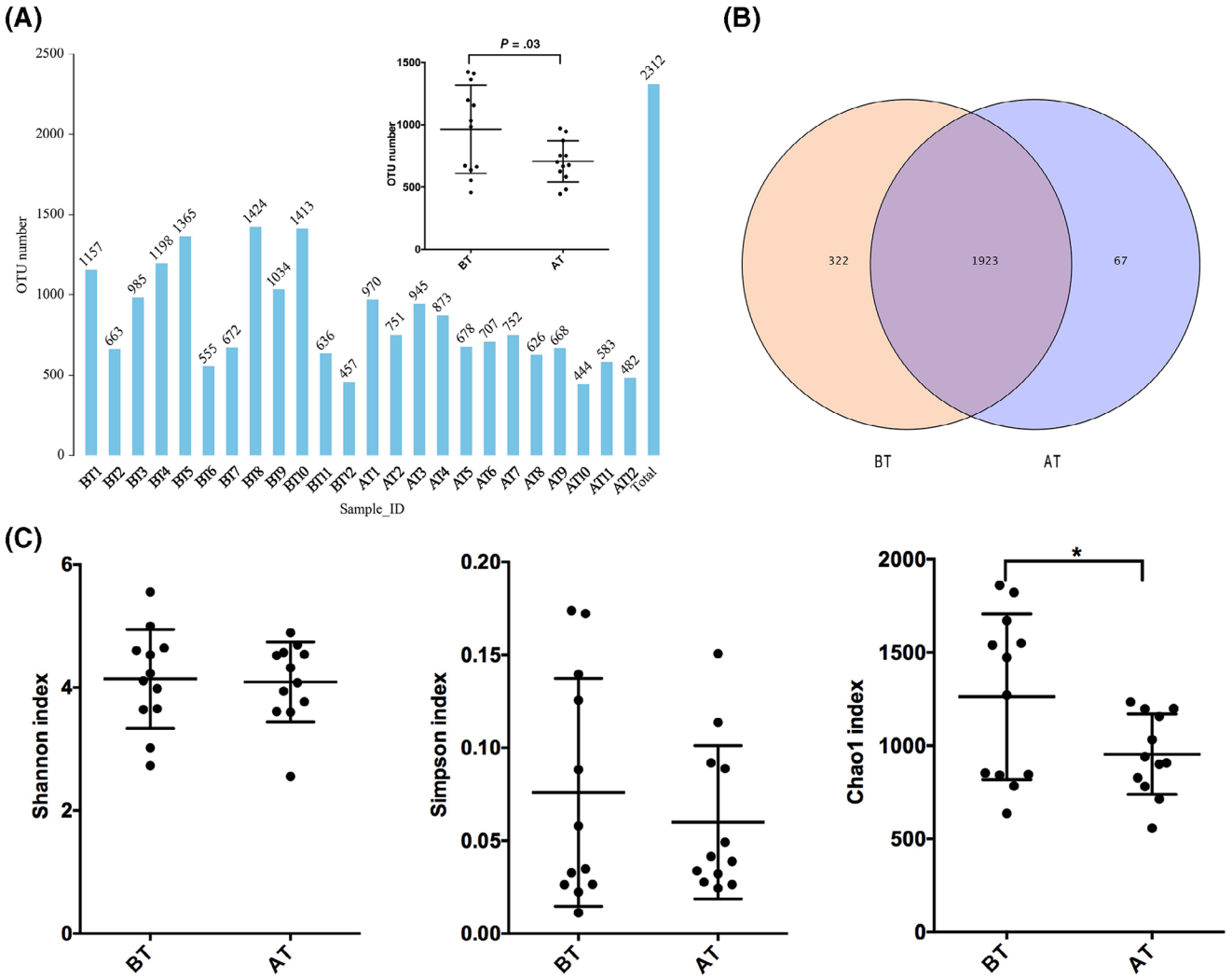

FIGURE 2 Operational taxonomic unit (OTU) analysis and alpha diversity indices of the healthy donkey fecal bacterial microbiota between before (BT) and after transport (AT) groups. A, The vertical axis (OTU number) represents the final OTU number after taxonomic analysis. B, Venn diagram of OTUs. The overlap section represented the shared OTUs between BT and AT group. C, Shannon index, Simpson index, and Chao1 index of the fecal bacterial microbiota between BT and AT group. Horizontal line represents the mean, and error bars represent the standard deviation (SD). *P < .05

transportation (AT), diet and water were not changed, and all donkeys were stabled with daily access to hay and water. The donkeys were housed in different areas of the same barn, without any contact with other animals. The same feeding methods and times were used BT and AT. Fodder was transported from the original location, thereby minimizing the effects of environment and food on the study.

2.2 | Sample collection

All samples were collected from each donkey within 30 minutes BT and AT. Five milliliters of blood was collected from the jugular vein. Blood samples were placed on ice, immediately transferred to the laboratory for analysis, and centrifuged at 3000*g* for 20 minutes at 4°C. The supernatants were stored in microtubes at −80°C until analysis. All laboratory analyses were performed within 24 hours. Fecal swabs, autoclaved and 15 cm long, were collected from the rectum (n = 12), stored in the microfuge tube, and frozen at −80°C pending DNA extraction. After sampling, all donkeys were housed at the breeding farm to provide genetic material (ie, semen) as select breeders (ie, Jackass) to be used either for breeding or as a germplasm reservoir (ie, frozen semen).

2.3 | Plasma concentrations of hormones and HSP90 analysis

Cortisol (Cor), heat-shock protein 90 (HSP90), and ACTH were measured using an ELISA-based techniques with commercial kits of Enzyme-linked Biotechnology (Shanghai Enzyme-linked Biotechnology Co., Ltd., China).

2.4 | DNA extraction and pyrosequencing

Total bacteria DNA was extracted from the fecal samples stored at −80°C using a genomic DNA extraction kit (Tiangen Company, Beijing, China) according to the manufacturer's protocol. The quality and concentration of the extracted DNA were measured using a NanoDrop spectrophotometer (ND-1000, NanoDrop Technologies, Wilmington, DE). The V3 and V4 regions of the 16S rRNA gene were amplified by PCR (95°C for 5 minutes, followed by 25 cycles of 95°C for 30 seconds, 50°C for 30 seconds, 72°C for 40 seconds, and 72°C for 7 minutes) using specific bacterial primers (forward: 5′-ACTCCTACGGGAGGCAGCA-3′, reverse: 5′-GGACTACHVGGGTW TCTAAT-3′). Indexed adapters were added to the ends of the primers. The PCR products were mixed with the same volume of 2× loading buffer and subjected to 1.8% agarose gel electrophoresis for detection. Samples with a bright main band of approximately 450 bp were chosen and mixed in equidensity ratios. Then, the mixture of PCR products was purified using a GeneJET Gel Extraction Kit (Thermo Fisher Scientific, Waltham, Massachusetts). Sequencing libraries were validated using an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, California) and quantified using a Qubit 2.0 Fluorometer (Thermo Fisher Scientific). Finally, paired-end sequencing was conducted using an Illumina HiSeq 2500 platform (Illumina, San Diego, California) at Biomarker Technologies Co., Ltd (Beijing, China).

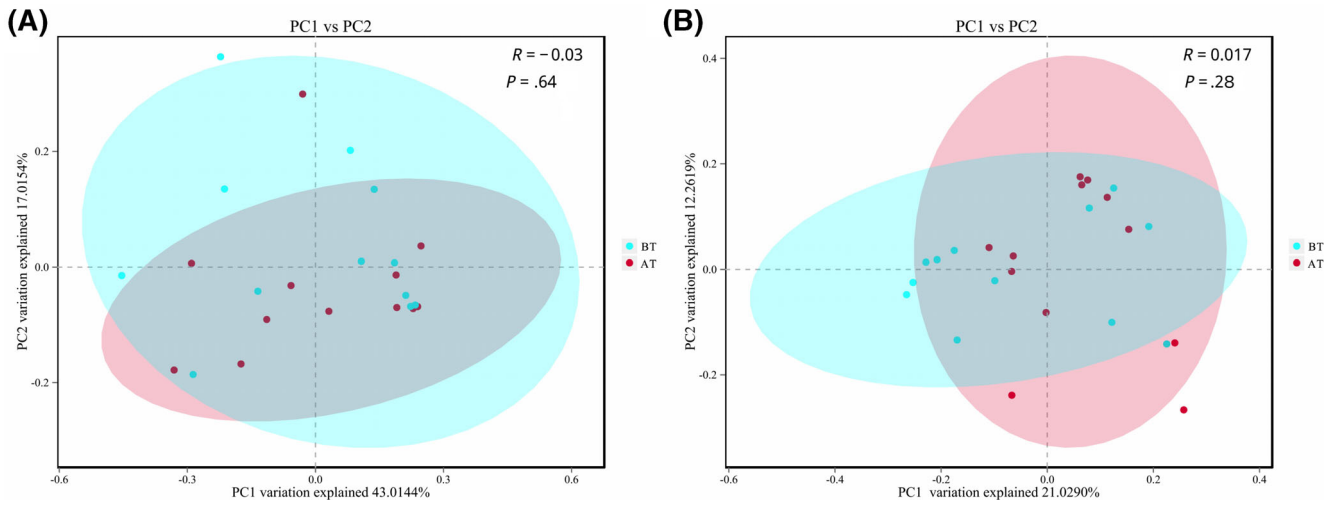

FIGURE 3 Principal coordinate analysis (PCoA) analysis between before (BT) and after transport (AT) groups based on the weighted UniFrac (A) and unweighted UniFrac (B) algorithms. UniFrac PCoA were calculated based on a 97% OTU similarity to show overlap between BT and AT microbiota. Percentage values at the axes indicate contribution of the principal components to the explanation of total variance in the dataset. Weighted UniFrac PCoA (A) shows the first two PCs (PC1 explaining 43.01% of the variance; PC2 explaining 17.02% of the variance). Unweighted UniFrac PCoA (B) shows the first two PCs (PC1 explaining 21.03% of the variance; PC2 explaining 12.26% of the variance). Each sample of BT group (n = 12) is represented by a point in blue and AT group (n = 12) in red. The weighted and unweighted UniFrac distances were compared using analysis of similarities (ANOSIM)

2.5 | Bioinformatics and data analysis

The raw paired-end reads from the original DNA fragments were merged using FLASH v1.2.11 and assigned to each sample according to the unique barcodes. QIIME[18] (version 1.8.0) UCLUST[19] software was used based on 97% sequence similarity. The tags were clustered into operational taxonomic units (OTUs). Alpha diversity index was evaluated using MOTHUR software (version v.1.30). The number of sequences contained in each sample was standardized as previously described[15] to compare alpha diversity indices among the samples. Analysis measures included Shannon, Chao1, and Simpson indexes. For beta diversity analysis, principal coordinate analysis (PCoA) between groups based on the unweighted UniFrac and weighted UniFrac algorithms was performed. Analysis of similarities (ANOSIM) was carried out based on unweighted and weighted UniFrac distance to assess the difference of microbial community structures between AT and BT groups. Linear discriminant analysis (LDA) effect size (LEfSe) analysis, using the nonparametric factorial Kruskal-Wallis sum-rank test and unpaired Wilcoxon rank-sum test, was performed to identify differentially abundant OTUs with 97% sequence similarity between groups online (http://huttenhower.sph.harvard.edu/galaxy).[20] The LEfSe scores measure the consistency of differences in relative abundance between taxa in the groups analyzed (BT vs AT), with a higher score indicating higher consistency. Taxa with LEfSe score >2 and $P < .05$ were considered significant. The Kolmogorov-Smirnov test was used for testing normality. P values were calculated using a Student's paired t test using GraphPad Prism software, and a P value of <.05 was considered significant.

3 | RESULTS

3.1 | Transportation of donkey alters plasma concentrations of hormones and HSP90

Results showed significant increases in plasma ACTH (163.8 ± 31.9 to 315.8 ± 27.9 pg/mL, $P = .0001$), Cor (58.1 ± 14.6 to 71.1 ± 9.60 ng/mL, $P = .04$), and HSP90 (10.8 ± 1.67 to 14.6 ± 1.75 ng/mL, $P = .02$) concentrations on the day of arrival compared with concentrations on the day before transportation (Figure 1).

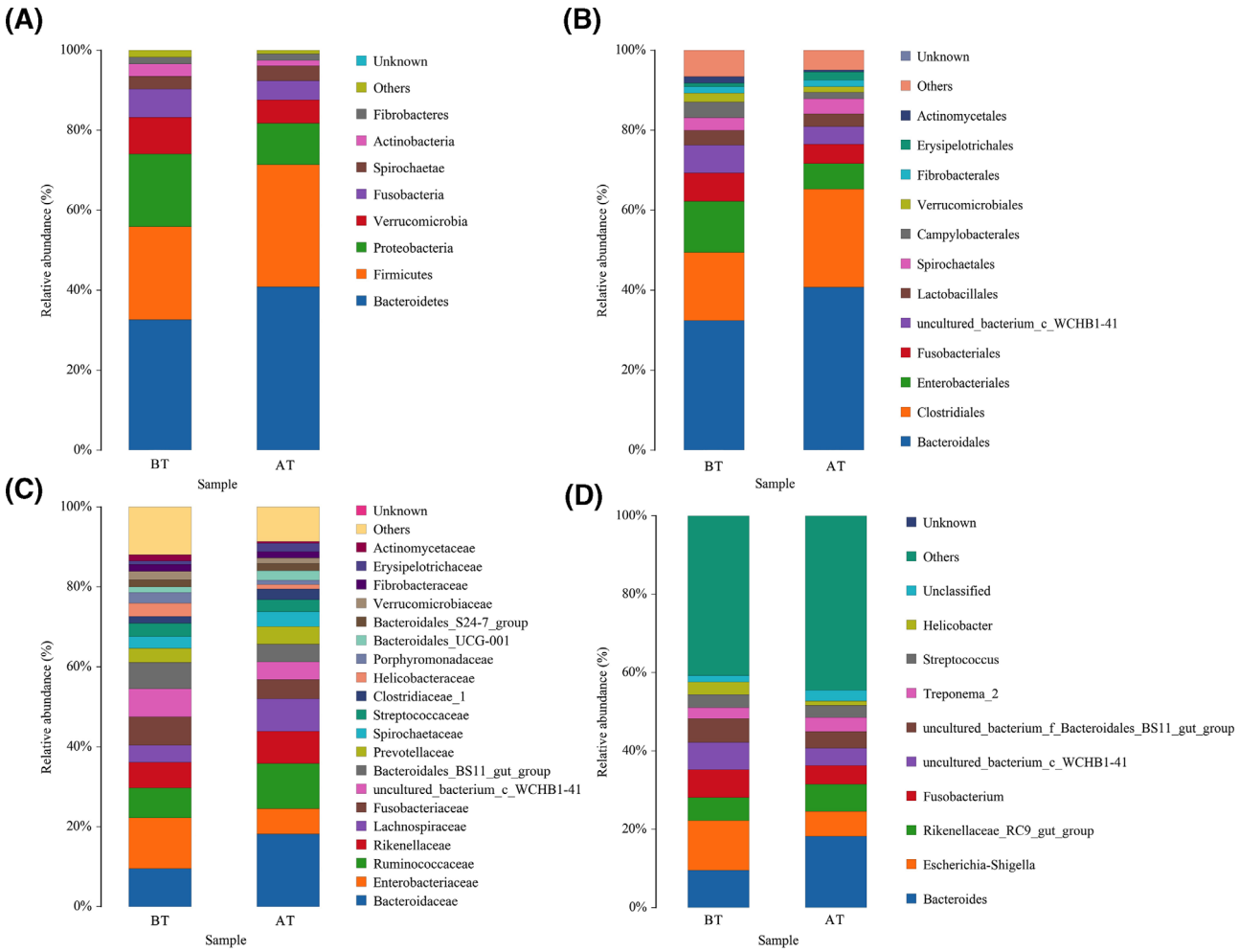

FIGURE 4 Relative abundance of predominant phyla (A), orders (B), families (C), and genera (D) in before transport (BT) (n = 12) and after transport (AT) (n = 12) groups. Other: Bacterial taxa with ≤1% abundance, Unknown: Sequences which could not be classified

TABLE 1 Linear discriminant analysis of differentially abundant bacterial taxa between before/after transport (BT/AT) groups

Bacterial taxa	Group	LDA (Log10)	*P* value
f__Coriobacteriaceae	BT	3.04	.01
g__Atopostipes	BT	2.80	.03
g__Jeotgalicoccus	BT	2.76	.02
g__Family_XIII_AD3011_group	BT	2.60	.006
g__Sporosarcina	BT	2.49	.04
o__JG30_KF_CM45	BT	2.37	.03
g__Ornithinimicrobium	BT	2.35	.04
g__Eubacterium__ruminantium_group	BT	2.25	.03
g__Pseudomonas	BT	2.20	.02
g__Eubacterium	BT	2.16	.04
g__Sarcina	BT	2.16	.02
f__Moraxellaceae	BT	2.14	.02

Note: Taxonomic levels are represented as o (order), f (family), and g (genus).

3.2 | Sequencing quality data and alpha diversity analysis

A total of 1 233 776 pairs of reads were obtained from the 24 samples that were sequenced. Double-end read splicing and filtering resulted in 1 075 841 clean tags, and each sample produced 44 827 clean tags on average. The number of OTUs in fecal samples AT (AT1-12) decreased markedly ($P = .03$) relative to that BT (BT1-12; Figure 2A). A Venn diagram showing the number of shared OTUs between AT and BT groups is presented in Figure 2B, and the Chao1, Simpson, and Shannon indices were calculated (Figure 2C). A significant decrease in Chao1 index ($P = .04$) was found in the fecal samples AT compared with that BT. The lower Chao1 index in the AT group indicated that transportation stress might decrease the bacterial richness. The other alpha diversity indices (Simpson diversity index and Shannon evenness index) were not significantly different between AT and BT groups.

3.3 | Beta diversity analysis

The PCoA diagrams did not show separation between the samples in BT and AT group (Figure 3). The ANOSIM of weighted UniFrac and unweighted UniFrac distances showed that this clustering was not significant (weighted UniFrac: $R = -0.03$, $P = .64$; unweighted UniFrac: $R = 0.017$, $P = .28$), indicating no significant difference in the bacterial composition between BT and AT.

3.4 | Phylogenetic analysis

Eight phyla, 12 orders, 20 families, and 9 genera had mean relative abundance >1% (Figure 4). The predominant phyla in each group were *Bacteroidetes* (BT median: 32.6%, minimum: 10.1%, maximum: 50.8%; AT median: 40.9%, minimum: 27.7%, maximum: 53.3%), *Firmicutes* (BT median: 23.3%, minimum: 11.3%, maximum: 38.3%; AT median: 30.5%, minimum: 19.6%, maximum: 58.7%), *Proteobacteria* (BT median: 18.1%, minimum: 0.7%, maximum: 36.6%; AT median:10.3%, minimum: 0.8%, maximum: 27.4%), and *Verrucomicrobia* (BT median: 9.1%, minimum: 1.0%, maximum: 18.7%; AT 5.8%, minimum: 1.7%, maximum: 15.2%). Within *Bacteroidetes*, the most abundant families were *Bacteroidaceae* (BT median: 9.5%, minimum: 0.04%, maximum: 32.3%; AT median: 18.2%, minimum: 0.01%, maximum: 37.2%) and *Rikenellaceae* (BT median: 6.3%, minimum: 0.9%, maximum: 13.8%; AT median: 8.1%, minimum: 0.2%, maximum: 19.3%). *Enterobacteriaceae* (BT median: 12.7%, minimum: 0.02%, maximum: 34.1%; AT median: 6.3%, minimum: 0.02%, maximum: 15.3%) was the most abundant family within *Proteobacteria*. *Ruminicoccaceae* (BT median: 7.5%, minimum: 2.8%, maximum: 16.5%; AT median: 11.3%, minimum: 3.1%, maximum: 34.1%) was the most abundant family within *Firmicutes*, followed by *Lachnospiraceae* (BT median: 4.3%, minimum: 1.0%, maximum: 11.9%; AT median: 8.1%, minimum: 3.1%, maximum: 22.2%).

The LDA score indicates the effect size and ranking of each bacterial taxon. In our study, LEfSe analysis identified a large number of bacterial taxa (eg, *Eubacterium*, *Atopostipes*, and *Pseudomonas*) with significantly higher abundance in the BT group than in the AT group. No taxa were enriched in AT group relative to BT group. Differentially abundant taxa within these 2 groups are listed in Table 1.

4 | DISCUSSION

In our study, the effect of transport on the phylogenetic composition of donkey fecal microbiota was analyzed. Differences in the relative abundances of phyla, classes, and orders and loss of bacterial diversity and richness were observed.

4.1 | Effects of transportation on donkeys

The ACTH and Cor concentrations increased under stress in response to changes in the external environment. These hormones are important indicators of the stress reaction of animals, including beef cattle,

piglets, chicken, and horses. During stressful situations, such as transportation, ACTH and cortisol concentrations in plasma increase variably.[21-23] Heat shock protein 90 is an important stress protein in organisms because it is rapidly activated and synthesized during stress.[24] Previous studies showed that transport stress increases the heat shock protein concentrations in pigs.[25,26] In our study, ACTH, Cor, and HSP90 concentrations increased significantly AT, and this finding is in agreement with other studies worldwide. For example, ACTH and cortisol concentrations respectively increased to 4.9-fold and 1.8-fold above baseline after beef cattle transportation.[27] Similar to the above study, plasma cortisol concentrations in transported horses increased markedly.[22] Therefore, we speculated that environmental disturbances (ie, cold weather, overcrowding, bumpy transportation) serve as stress factors, triggering a stress response in donkeys.

4.2 | Microbiota differences between BT and AT

Bacterial species diversity and richness are important factors in gastrointestinal health. A recent study showed that donkeys have a rich, diverse, and multifunctional microbiota along the gastrointestinal tract.[28] We found that the Chao1 index was significantly decreased AT ($P = .04$), but the Shannon and Simpson indices were not changed, suggesting that transportation results in a low level of bacterial richness but does not alter diversity and community evenness. Previous studies showed that the intestinal flora of healthy animals can regulate immune function and the intestinal mucosa's barrier function by producing lactic acid and short chain fatty acids (SCFAs),[29] and inhibit the adhesion of pathogens on the intestinal wall to prevent the occurrence of an inflammatory reaction.[30,31] Having a diverse microbiota is beneficial for promoting host immune defenses and metabolism,[32] and loss of species diversity and richness have been associated with several gastrointestinal diseases, including diarrhea,[33,34] acute enteric infections,[35] and colitis in adult horses.[36] Thus, we hypothesized that the decreased bacterial richness induced by transport stress might decrease resistance to infectious agents, making the donkeys more susceptible to disease, and cause a series of gastrointestinal problems AT, such as diarrhea, which is consistent with earlier findings.[34,37,38] The diversity indices of the fecal microbiota do not differ among horses experiencing colitis,[11] transport, fasting, anesthesia[15] as compared to controls. These results suggest that the decreases in bacterial diversity AT might not play a similar role in donkey disease development, and bacterial richness might be an important factor in this phenomenon.

Transport stress rapidly affects the composition of gut microbiota and host physiology by the generation of bioactive metabolites.[15,27] In our study, LEfSe analysis identified that AT, significant decreases were found for some SCFAs-producing bacteria, including *Eubacterium* genus and *Coriobacteriaceae* family.[39] The SCFAs are not included in the diet but synthesized by colonic commensal bacteria from dietary carbohydrate and are important for intestinal health.[40] Interfering with the SCFA synthesis in the colon may result in diarrhea because increased production of SCFAs enhances colonic fluid production and corrects dehydration associated with acute diarrhea.[41] A previous study found *Eubacteriua* were significantly lower during and immediately after diarrhea than during a diarrhea-free period of normal health in children.[42] The number of *Coriobacteriaceae*, found abundantly in the healthy gut, was significantly decreased in colitis cases.[43] In addition to the decrease in SCFAs-producing bacteria, significantly low richness of *Streptococcus*, *Atopostipes*, and *Pseudomonas* was observed AT. *Atopostipes* and *Pseudomonas* are bacteria that produce branched fatty acids (BCFAs), which also have an important influence on intestinal health and are related to various health conditions. These compounds are metabolized by enterocytes and have a beneficial role against inflammation in the upper intestinal tract, alter the microbiota, and increase the expression of anti-inflammatory cytokines.[44] *Streptococcus* is identified as an enriched taxon using LEfSe in healthy horses[38] and negatively correlated with inflammatory variables (tumor necrosis factor-α [TNF-α], lipopolysaccharide [LSP], and H_2O_2 yield).[45] A previous study showed that *Streptococcus thermophilus* could protect the intestinal tract and improve colonic inflammation in experimentally-induced inflammatory bowel disease in rats.[46] Furthermore, *S thermophilus* also can decrease the secretion of some inflammatory factors by increasing the secretion of folate, which regulates the immune status.[47] The decrease in the relative abundance of members of these families AT might interfere with metabolite synthesis, which may increase the rate of inflammatory bowel disease during recovery. Additional studies could focus on these bacterial families to develop preventative or therapeutic measures.

4.3 | Limitations

Our study had some limitations, including limited sample size, animals of only a single sex and inevitable environmental factors. Previous studies have found that potential confounding factors, such as age,[38] sex,[48] diets,[49] and environmental change[50] could impact the bacterial microbiota of different body sites.

Thus, we have taken a series of measures to minimize the effects of these factors on the experiments, for example selecting donkeys of the same sex and age, as well as consistency in fodder and feeding management. Environmental change is most likely to impact the intestinal microbial composition in our study because of the changing locations where the donkeys were housed. We cannot avoid environmental bacteria being present in the intestinal microbiota, but environmental bacteria have been shown to be a minimal component of the intestinal microbiota in cattle.[51] Additionally, a limitation of our study is the absence of a control group that remained on the farm before transportation to show that the observed changes in the microbiota are caused by transport, rather than change over time. However, because most Chinese farmers purchase donkeys during winter for breeding in the following spring, long-distance transportation mainly occurs during winter. Our study was performed in dry and cold climatic conditions, and therefore our results are not likely applicable to donkeys transported under other environmental conditions,

such as long-distance transportation in hot, humid weather. Another consideration is that fecal microbial composition does not fully reflect the microbial composition of different regions in the gastrointestinal tract. Additional studies about the effects of transport stress on the microbiota of various intestinal segments of the donkey are required.

ACKNOWLEDGMENT

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CONFLICT OF INTEREST DECLARATION

Authors declare no conflict of interest.

OFF-LABEL ANTIMICROBIAL DECLARATION

Authors declare no off-label use of antimicrobials.

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE (IACUC) OR OTHER APPROVAL DECLARATION

Approved by the Ethics Committee for Laboratory Animal Care (Animal Ethics Procedures and Guidelines of China) at the Shenyang Agricultural University (Permit No. 264SYXK<Liao>2011-0001, September 2018).

HUMAN ETHICS APPROVAL DECLARATION

Authors declare human ethics approval was not needed for this study.

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